THE SIZE OF THE GUANOSINE TRIPHOSPHATE POOL AS A FUNCTION OF STEADY STATE GROWTH RATES IN ESCHERICHIA COLI

A THESIS

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

BY

JOHN ROSS

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

MAY, 1974

R- x R-5A

ABSTRACT

BIOLOGY

ROSS, JOHN B.S., Morehouse College, 1968

The Size of the Guanosine Triphosphate Pool as a Function of Steady State Growth Rate in Escherichia coli

Advisor: Dr. Thomas Norris

Master of Science degree conferred May 20, 1974

Thesis dated May, 1974

In an effort to understand how the rate of RNA synthesis is controlled at different steady state growth rates, this investigation has examined the correlation between nucleotide pool size and the rate of total RNA synthesis at different growth rates.

Escherichia coli cells were grown in minimal media in chemostats at growth rates corresponding to doubling times of 1.5 and ten hours. The cells were extracted with formic acid and the extract was spotted on polyethyleneimine thin layer chromatography plates. After a two-dimensional development, autoradiographic procedures were used to identify location of nucleotides which were subsequently quantitated by removing the spots and counting.

The results of this investigation revealed that there is a 1.66-fold difference in the size of the guanosine triphosphate pool over the growth rate span studied. These results correlate with the 1.7-fold difference in the total rate of RNA synthesis over the same growth span.

The results of this paper also support the views that ribosomal RNA is unstable for slow growing cultures and that the control of the three species of RNAs is non-coordinate.

ACKNOWLEDGMENTS

The author wishes to thank Drs. Norris, Lumb and Myers for serving on his committee.

Special thanks go to Dr. Norris, the major professor for his great help throughout the tenure of this research. In addition, Dr. Norris has shown the author new and fruitful ways of looking at the world.

The author wishes to express his gratitude to Matthew George, Bobby Ledpart and Heuldine Webb for helping to perform experiments and aiding in many useful discussions.

The author gratefully acknowledges the generous monetary support of BISRIP assistantship.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

The mechanisms which govern the rate of total ribonucleic acid synthesis as well as the rate of RNA accumulation are being investigated with emphasis on methodology and a re-evaluation of theoretical concepts. With the use of anion exchange polyethyleneimine thin layer chromatography (Randerath and Randerath, 1964), nucleoside triphosphates have become relatively easy to isolate and assay. Furthermore, a re-evaluation of RNA synthesis has been developed by Nierlich (1968). According to Nierlich, early investigators have interpreted net RNA synthesis (rate of synthesis of rRNA and tRNA) as a measure of total RNA synthesis (rate of synthesis of mRNA, rRNA and tRNA). In addition, Koch (1968) and Bremer and Mueller (1968) indicated that the kinetics of labelling of RNA as determined by use of radioactive precursors were being widely misinterpreted and consequentially they devised a new mathematical approach for the analysis of kinetic data.

The purpose of this research was to investigate whether a correlation exists between the size of the guanosine triphosphate pool and the total rate of RNA synthesis employing the sensitive methodology and new theoretical concepts described above.

The bulk of the literature, both early and recent, appears not to support a regulatory role for nucleoside triphosphates. However, the results of this investigation clearly contradict this view as the results suggest that the GTP pool is indeed a regulatory device for

the total rate of RNA synthesis.

In addition, Norris and Koch (1972) postulated that rRNA as well as tRNA was unstable for cultures growing at a very slow growth rate. The results of this paper clearly support this view and suggest further that the rate of synthesis of the three classes of RNA's are controlled independently.

Finally, Norris and Koch (1972) emphasized that their data pertaining to the total rate of RNA synthesis was subject to certain limitations since they didn't know whether beta-galactosidase synthesis was governed by an average messenger molecule. However, since the data presented in this paper pertaining to the total rate of RNA synthesis is consistent with the date reported by Norris and Koch (1972), it would follow that beta-galactosidase could be considered an average messenger molecule for cells of Escherichia coli B.

CHAPTER II

REVIEW OF LITERATURE

General

The control mechanisms governing RNA synthesis in bacteria have been under investigation for the past 15 years. As early as 1952 it became apparent that for a variety of cell types there is a general correlation between the rate of growth and the cell's content of RNA. For example, when cells of Escherichia coli K-12 are deprived of any required amino acid, the net rate of ribonucleic acid synthesis decreases to a few per cent of its normal value (Sands and Roberts, 1952; Pardee and Prestidge, 1956; Gros and Gros, 1958; Stent and Brenner, 1961; Neidhardt, 1963). Furthermore, the rate of RNA accumulation is also severely reduced when transferring cells from a nutrient medium supporting a faster growth to one supporting a slower growth (Kjeldgaard, Maaloe and Schaechter, 1958; Neidhardt and Fraenkel, 1961).

In contrast, other E. coli, also of the K-12 strain, were found to continue net RNA synthesis during amino acid starvation (Borek, Ryan and Rockenbach, 1955). Stent and Brenner (1961) coined the genotypic symbols RC^{str} and RC^{rel} and the phenotypic terms stringent and relaxed respectively for those strains which did and did not restrict their RNA synthesis during amino acid starvation. The RC in these terms stands for RNA control. At present, rel^- is used for RC^{rel} and rel^+ for RC^{str} (Taylor and Trotter, 1967).

Various parameters such as protein synthesis (Neidhardt and Fraenkel, 1961), amino acid uncharged transfer RNA molecules (Stent and

Brenner, 1961; Kurland and Maaloe, 1962), polysome complexes (Ron, Kohler and Davis, 1966; Morris and DeMoss, 1966), and polyamines (Rania, Jansen and Cohen, 1967) have been investigated as possible control mechanisms for RNA synthesis.

In order to correlate the above parameters with RNA synthesis, investigators induced changes in the growth rate of the organism which subsequently produced changes in RNA synthesis and correlated these changes with those of RNA synthesis. Thus, it was equally important during that time for researchers to find the actual relationship between RNA synthesis and the growth rate of the bacterium.

Koch (1965) found that during a shift up (sudden nutritional enhancement) of a bacterial culture there was a very rapid change in net RNA synthesis.

Koch (1968) and Norris (1970) found that the ratios of the rate of RNA accumulation per unit amount of DNA varied from 1 to 3 for cultures growing at doubling times of 0.5 hours and 24 hours respectively. They also found that during that same span of growth rates the ratio of DNA per unit amount of protein remained constant. Then through mathematical manipulations, Koch and Norris (1973) showed that the rate of accumulation of RNA per unit amount of DNA for a culture growing at a doubling time of 0.5 hours was 144 times faster than that same rate for a culture doubling every 24 hours. Furthermore, they showed that the rate of protein accumulation per unit amount of RNA for a culture doubling at 0.5 hours was 16 times faster than that same rate for a culture doubling every 24 hours. It should be pointed out that the early studies involving control mechanisms for RNA synthesis as well as a bulk of the recent literature did not employ such a wide variation

in growth rates as those designed by Norris (1970).

Nevertheless, early investigators correlated different parameters of RNA control with the rate of RNA synthesis at different, but not vastly extreme, growth rates. If the particular parameter under investigation changed proportionally with the rate of RNA synthesis as the growth rates were changed, it would be considered as a possible control mechanism for RNA synthesis.

However, neither of the four parameters mentioned previously have shown a direct relationship with the rate of RNA synthesis.

A fifth parameter, nucleoside triphosphates, was also investigated as a possible control mechanism of RNA synthesis. Since this parameter is the major issue of this paper it will be discussed in more detail.

Since ribonucleoside triphosphates were the substrates for RNA polymerizations, the simple possibility of a substrate level regulation of the rate of RNA synthesis by triphosphates required examination. Neidhardt and Fraenkel (1961) reported changes in triphosphates during nutrient-induced growth shifts. Furthermore, correlation between triphosphate pools and cellular growth rates was established by Franzen and Binkley (1961); and Smith and Maaloe (1964). Nevertheless, the bulk of the early literature appears not to support an important regulatory role for the triphosphates (Goldstein, Brown and Goldstein (1960); Edlin and Neuhard (1967)). These early studies, however, did not give sufficiently detailed resolutions of the triphosphate changes to permit an assessment of their role in RNA synthesis. With recent developments in methodology, a more extensive analysis of the nucleoside triphosphate changes, as required for an improved evaluation of their possible regulation of cellular RNA synthesis, has become feasible (Randerath and

Randerath, 1964).

With the employment of new methodology, nucleoside triphosphates as a control of RNA synthesis received recent attention when Gallant and Cashel (1967) showed that in plasmolyzed cells there is an amino acid dependent step in the synthesis of nucleoside triphosphates. Furthermore, Cashel and Gallant (1968) and Gallant and Harada (1969) were able to show that changes in the nucleoside triphosphate pool are positively correlated with changes in the rate of RNA synthesis that occur during a nutritional shift. In addition, they discovered a tetraphosphate of guanosine (5' diphosphate, 2' or 3'-diphosphate guanosine) (Cashel and Gallant, 1969) whose kinetics of formation and disappearance are directly correlated with the onset and disappearance of the stringent phenotype. This compound may regulate RNA synthesis (Cashel, 1969; Cashel and Kalbacher, 1970). They further postulated that ppGpp (MS I for Magic spot I) whose rapid accumulation in response to amino acid starvation is governed by the RC gene product, inhibits IMP dehydrogenase and adenylosuccinate synthetase, the first enzymes of the guanylate and adenylate pathways (Gallant, Irr and Cashel, 1971). While there has been neither proof nor disproof of the effects of the tetraphosphate, there is contradictory evidence in regard to correlation of pool size with RNA synthesis.

Edlin and Neuhard (1967) did not find any abrupt decrease in the nucleoside triphosphate levels upon starvation of stringent cells. In relaxed cells, they found little change in the pool levels until the net rate of RNA synthesis began to double or triple. Later, Edlin and Stent (1969) also found no decrease in the triphosphate pool levels

during amino acid starvation of a stringent strain, while Bagnara and Finch (1968) found that the pool levels increased. Wong and Nazar (1972) observed that during growth rate shifts obtained by changing carbon or nitrogen source or by adding inhibitors, changes in nucleoside triphosphate levels in all instances were found to be inadequate to account fully for the observed preferential inhibition of stable RNA accumulation.

All of these theories reflect the general belief that the synthesis of all species of RNA, messenger, transfer and ribosomal, are equally subject to the same control. Nierlich (1968) re-evaluated amino-acid control over RNA synthesis. He suggested that while studying the kinetics of labelling RNA molecules, investigators have compared certain parameters with the net rate of RNA synthesis and not the total rate of RNA synthesis. His results suggest further that the synthesis of RNA is non-coordinate and that during amino acid starvation messenger RNA, but not ribosomal and transfer RNA is being made. Therefore, a measurement of mRNA becomes necessary if one is to measure the total rate of RNA synthesis. Since this RNA is unstable, it is detectable only by procedures which bypass the problem imposed by the preferential reutilization of degradation products of the unstable RNA in RNA synthesis.

Support of the theory of non-coordinate control was recently given by Norris and Koch (1972). They determined the relative amounts and relative rates of synthesis of messenger, ribosomal and transfer RNA in Escherichia coli strain B at doubling times ranging from 55 minutes to 10 hours. They found that mRNA represented a constant fraction (0.03) of the amount of total RNA at all growth rates, and the ratio of the rate of synthesis of mRNA to rRNA increases twofold while the tRNA to

rRNA ratio decreases twofold as the growth rate is decreased about tenfold. Coffman, Norris and Koch (1971) had previously found that the effective messenger life and the chain elongation rate of mRNA were the same throughout these ranges of doubling times. Based on this experimental evidence they postulated that at slow growth rates 16 + 23s rRNA is turning over and a metabolic control is affecting the rate of RNA synthesis by acting on the frequency with which transcription is initiated.

Furthermore, they suggested that since the ratios of the velocities of synthesis of m-, r- and t-RNA are significantly different for the 1 and 10 hour doubling cultures, then the control of these three classes of RNA must be independently mediated. For a more precise analysis, they found that over the growth rate range studied, mRNA constitutes approximately 0.03 of the total RNA at all growth rates, while 16 + 23s rRNA constitutes 0.80, 0.77 and 0.73 and tRNA constitutes 0.15, 0.20, and 0.24 of the total RNA at doubling times of 55 minutes, 5 and 10 hours respectively. The mRNA is synthesized at 0.55, 0.63 and 0.71 of the total rate of synthesis of all RNA species at doubling times of 55 minutes, 5 hours and 10 hours respectively. The relative rates of synthesis of 16 + 23s rRNA are 0.35 and 0.22 and tRNA are 0.08 and 0.03 at doubling times of 55 minutes and 10 hours respectively. From these proportions, Norris and Koch (1972) postulated that the amount of mRNA degraded per unit amount of DNA and per unit time can be simply obtained by dividing by the average chemical life of the messenger fraction, or by the half-life divided by 0.693.

In a previous paper, Coffman, Norris and Koch (1971) showed that the lac messenger molecules have the same average life span over this

range of growth condition. Thus, Norris and Koch (1972) concluded that if all messenger molecules have this same average life span of 230 seconds at 37 C, then the rate of mRNA degradation varies from 0.039 to 0.023 RNA g/g DNA minute as growth is slowed from 1 hour to 10 hours.

Nierlich (1972) measured the total rate of RNA synthesis in E. coli ML-30 under steady state conditions and transition shifts. He found that in differing steady states of growth, cells vary not only their total rate of RNA synthesis, but also the fraction of the total given to the formation of unstable species. This was confirmed by Winslow and Lazzarini (1970). Nierlich's data show that the ratio of the rate of synthesis of stable to unstable RNAs was 1 to 1 in minimal -glucose and 1 to 2 in enriched medium. Moreover, he observed that transients between steady states give strong indications that the synthesis of stable and unstable RNA species can vary independently. In shift up for example, while the net rate of accumulation, presumably largely the synthesis of the stable ribosomal and tRNA's, increases immediately, the rate of total synthesis increases only in the course of 5 to 15 minutes. Nierlich proposed that the mechanism for the control of RNA synthesis involves both a means whereby the total capacity of the cell to make RNA is limited and a mechanism by which the distribution of that capacity between template sites leading to the synthesis of stable and unstable RNA species is regulated.

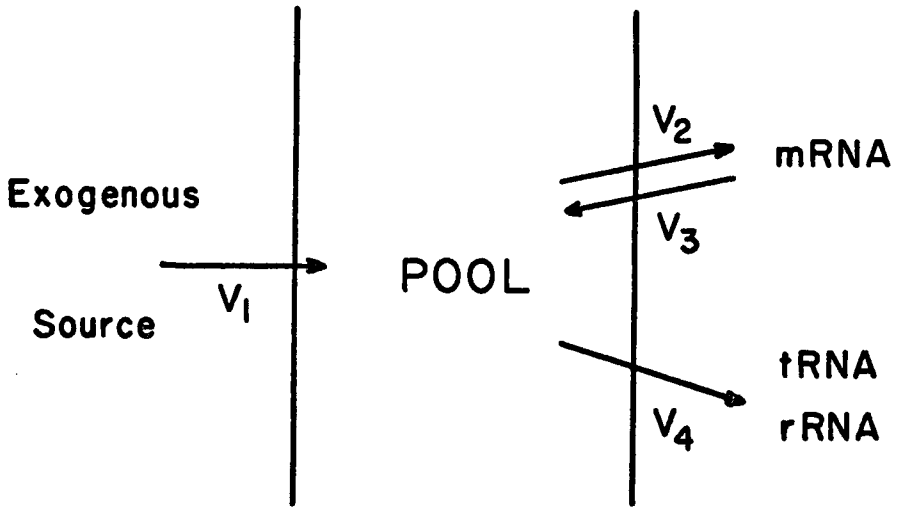
The work of various other researchers (Winslow and Lazzarini, 1969; Bremer and Mueller, 1968) are consistent with Nierlich's (1972) and Norris and Koch's (1972) data.

Bremer and Dennis (1973) showed that the chain elongation rates for rRNA are twofold higher than chain elongation rates reported for messenger RNA. From this data he suggested that, following a nutritional shift up, the transfer of a relatively small number of RNA polymerase molecules from unstable to stable RNA genes occurs to cause a rapid increase in the net rate of RNA synthesis. Furthermore, Bremer and Dennis indicated that the linear density of polymerase molecules on the ribosomal DNA template increases with the bacterial growth rate such that in rapidly growing bacteria, all ribosomal RNA genes (48 copies at a doubling time of 20 minutes) are fully saturated by RNA polymerase.

It thus appears that there is no single hypothesis that can explain all the data. In addition, most of the data used in defense of the various hypotheses cannot distinguish between what is cause and what is effect or separate correlations of cause and effect. Nevertheless, none of the data disproves that there is a correlation between total rate of RNA synthesis and the size of the nucleoside triphosphate pool. Since a measure of the total rate of RNA synthesis involves an account of mRNA breakdown, problems arise as to how the kinetics of labelling of RNA should be interpreted. This problem is considered in the next section.

Interpretation of Tracer Kinetic Data

From the beginning of the interest in the control mechanisms which govern RNA synthesis, measurements of that synthesis have been conducted under various conditions. The desired conditions have varied from amino acid starvation, steady state, exponential growth, sudden nutritional enhancement (shift-up) or sudden nutritional deprivation (shift-down). Then the increase or decrease in the rate of RNA synthesis due to transitions between shifts or different growth rates of the cultures is measured colorimetrically or an external radioactive precursor is added and the accumulation of that precursor into RNA is determined. The rate at which radioactivity accumulates in acid precipitable materials -- RNA and DNA -- is interpreted in various ways. Nierlich (1968), in his re-evaluation paper showed that this measure of the flow of radioactivity was not a measure of the total rate of RNA synthesis but a measure of the net rate of RNA synthesis. Schematically, the incorporation of an exogenous precursor into RNA may be represented as shown in Figure 1 (modified from Nierlich, 1968). The nomenclature is as follows (modified from Koch, 1968): mRNA stands for messenger RNA and sRNA for stable RNA, usually thought of as r- and t-RNA; P is the intracellular precursor pool and A is its exogenous source; the velocity, V_1 is rate of entry of nucleotides from the exogenous source into the pool; V_2 is the rate of utilization of nucleotides in the pool by m-RNA; V_3 represents the rate of return of nucleotides to the pool from m-RNA; and finally V_4 represents the rate at which pool nucleotides are utilized by stable RNA. The total rate of RNA synthesis is defined as $V_2 + V_4$. The net rate of RNA synthesis is defined as $V_2 - V_3 + V_4$.


Exogenous
Source
V_1
POOL
V_2
mRNA
V_3
tRNA
rRNA
V_4

To give the reader an idea about the relative magnitude of the velocities indicated, V_2 (the rate of synthesis of mRNA) is about 50% of the total rate of RNA synthesis ($V_2 + V_4$). Because messenger RNA constitute only 3% of the total RNA, the difference between V_2 and V_3 is very small. Consequently, if a radioactive precursor to RNA (such as radioactive guanine) is added to a culture and if the accumulation of the radioactivity into RNA is monitored over a long period of time, then the amount of radioactivity incorporated per unit amount of time is a measure of net RNA synthesis. However, what is not obvious is that the incorporation of radioactivity over short time periods (short with respect to the half-life of messenger RNA) has been shown to be proportional to net RNA synthesis. This, as shown experimentally by Nierlich (1968) and in theory by Koch (1968), results from the fact that in certain strains of E. coli, at least, there is no exchange of pool materials to the outside of the cell and the pool size, per unit amount of DNA, remains constant. It follows, therefore, that the only way a radioactive precursor can enter the pool from outside the cell is for a precursor to be permanently removed from the pool by forming part of a stable molecule. If a precursor molecule is removed into mRNA, because of the instability of this class of RNA molecules, a precursor will be returned to the pool from messenger breakdown. Since the pool size does not change, there is no mechanism for radioactivity from outside the cell to enter. This problem was not recognized by Koch in which he measured the incorporation of radioactive guanine into RNA at 20 second pulses and assumed that his measurements were

proportional to the total rate of RNA synthesis. Rather than measuring the total rate of RNA synthesis, Koch was actually measuring the net rate of RNA synthesis as he pointed out later (Koch, 1968).

The use of a radioactive precursor to RNA as a method of estimating the rate of RNA synthesis has another pitfall associated with it. Namely, if one adds a radioactive precursor, because the pool does not exchange with material on the outside of the cell, it takes a finite time for the specific radioactivity of the pool to reach that of the isotope outside the cell. This was shown by Winslow and Lazzarini (1969) in the case of UTP pools as is shown by this author for the case of GTP pools. Furthermore, for cells growing at different growth rates, the time course for equilibration of the pool may be growth rate dependent and, as well, the size of the pool itself may vary with growth rate.

Consequently, the kinetics of RNA synthesis, as monitored by the uptake of a radioactive precursor, require careful analysis. Such a careful analysis has been done by Bremer and Mueller (1968) and also more thoroughly by Koch (1968). The following treatment is taken from Bremer's analysis and has been modified by this author for the sake of clarity. The rate of uptake of radioactivity into RNA as a function of time, dR/dt is equal to $(\alpha_1 V_2 - \alpha_1 V_3 + \alpha_2 V_4)S$ for fast growing cultures in which ribosomal RNA does not turn over. dR/dt is equal to $(\alpha_1 V_2 - \alpha_1 V_3 + \alpha_2 V_4 - \alpha_2 V_5)S$ for slow-growing cultures where V_5 represents the velocity of degradation of ribosomal and transfer RNA. In the above equations, α represents the mole fraction of the particular nucleotide under investigation (in this case GTP) in RNA, and the lower case subscripts on the symbol represent the mole fraction in mRNA if 1, and

sRNA if 2. However, at short times (before there is a chance for mRNA or rRNA breakdown), dR/dt equals $(\alpha_1 V_2 + \alpha_2 V_4)S$ and at long times, $(\alpha_2 V_4 - \alpha_2 V_5)S$ for slow growing cultures where it has been demonstrated that ribosomal and transfer RNA are degraded (Norris and Koch, 1972). For fast growing cultures where r- and tRNA have been shown to be stable, dR/dt equals $(\alpha_2 V_4)S$.

Consequently, if one knows the specific activity of the pool as a function of time, S, and if the values α_1 and α_2 are known, then from dR/dt, the time course of radioactivity in RNA, one can calculate the velocities and therefore the total rate of RNA synthesis.

CHAPTER III

MATERIAL AND METHODS

Chemicals

Radioactive guanine was obtained from Schwarz-Mann BioResearch, Inc. For all cases (8-3H)guanine was used and the specific activities of two different batches were 20Ci/mmole and 12Ci/mmole. In experiments where calculations were dependent on the specific activity of (8-3H) guanine, this value was independently determined by isotopic dilution. Remaining chemicals were obtained from general laboratory suppliers and were used without further purification.

Bacteria and Growth Conditions

General.

The organism used was Escherichia coli strain B. In certain preliminary experiments a uracil deletion auxotroph of Escherichia coli strain B was used. According to Norris (1970) the above organism was isolated in 1965 by R. Peterson. Batch and chemostat cultures of these organisms were grown under forced aeration. The chemostat apparatus has been described by Norris (1970).

Media.

Cells were cultured in a low phosphate Tris-glucose medium (modified from Kaempfer and Magasanik, 1967). The low phosphate medium had the following composition and formulation: 0.08M NaCl, 0.02M KCl, 0.12M tris (hydromethyl) aminomethane, 0.001M $MgCl_2 \cdot 6H_2O$

and 0.0025M Na_2SO_4. At this point the media were neutralized with concentrated HCl to pH 7.3, then 0.02M NH_4Cl was added and the solution autoclaved. The mineral composition was 0.35mM $CaCl_2 \cdot 6H_2O$ and 0.002mM $FeSO_4 \cdot 7H_2O$ which was autoclaved separately. The Tris-glucose media x was stored as a 20X concentrate while the minerals were stored as a 500X concentrate. The carbon source, glucose, was stored as a 10% solution and diluted to give the batch culture a final concentration of 0.2% and the glucose limited chemostat a final concentration of 0.02%. A 0.2M KH_2PO_4 solution was autoclaved as a 100X concentrate and diluted to a final concentration of 2mM and used as excess phosphate, or diluted to a final concentration of 0.5 mM which does not limit growth for cultures whose turbidity is less than 0.5 absorbance units at 600nm (Hitachi Perkin Elmer 139 U.V.-visible spectrophotometer). The latter was used as limiting phosphate.

Growth conditions.

During experiments it was necessary to minimize culture manipulations during growth and sampling. Cooling, centrifugation, filtration and the like cause artifactual pool fluctuations (Cashel, 1973). Cell response times were too rapid to circumvent this problem by working quickly. Low cell densities (Absorbance at 600 nanometers less than 0.5) were employed to minimize transient anaerobiasis during sampling as well as to assure an excess of phosphate in the medium. Overnight cultures were prepared in 0.5 mM phosphate medium, with 0.2% glucose. Log phase growth was started by a tenfold dilution of the overnight culture into an

appropriate experimental medium. Experimental cell densities were generally from 0.2-0.5 absorbance units (600nm). At an absorbance of 0.5 ($2.5x10^8$ cells/ml) about 5% of the exogenous phosphate had been consumed and complete oxygen consumption during interruption of aeration takes at least one minute (Cashel, 1973).

Growth Problems of E. coli B in Low Phosphate Media

Cells of E. coli B were stored on slants containing nutrient broth. Previously, these cells were cultured in M-9 media (Roberts et al, 1957) containing 72mM KH_2PO_4.

When attempts were made to grow cells of E. coli B in Tris-glucose medium containing 2mM KH_2PO_4 and 0.2% glucose, difficulties were experienced in getting the cells to grow. Consequently, various experiments were conducted over a 4 month period to find the source of this problem. The experiments conducted were designed to test the effects of aeration, inoculum size, the age of the overnight culture used to initiate growth, extensive autoclaving of the cultural media and limiting nutrilites in the cultural medium on the growth pattern of the culture. The results indicated that neither of these parameters were inhibiting the growth of the culture.

Various lines of evidence, however, seem to indicate that bacterial strains must adapt to their environment. Thus a bacterial strain such as E. coli may not readily adjust from an environment where 72mM KH_2PO_4 is available to an environment where only 2mM or

5mM KH_2PO_4 is available. Subsequently, various trial and error experiments were conducted in varying phosphate concentrations in several different media. The results seem to suggest that environmental adaptability was indeed the reason for the initial observations of poor bacterial growth. To give the reader some idea as to how the cells were cultured, the problem of adaptability was approached in the following manner. Slant cultures of E. coli were first cultured in M-9 medium at 24 hour periods through three subcultures. Cells were then transferred to T-G (Tris-glucose) medium containing 40mM KH_2PO_4 and subcultured twice. Then the cells were sequentially transferred to T-G media containing 20, 5, 2 and finally 0.5mM KH_2PO_4; each sustaining cell densities greater than 1.0 absorbance units at 600nm with the exception of the latter whose phosphate concentration limits growth at 0.5 absorbance units. Liquid cultures were then stored in 0.5mM KH_2PO_4 and cultured as frequently as every three days. The cells were also periodically checked for contamination as well as mutation. It should be noted that the data presented here contradicts data reported by Shehata and Marr (1971). Their results indicate that tris (hydroxymethyl) aminomethane (Tris) buffer can be used only if the concentration of phosphate is greater than 10mM; at concentrations of phosphate less than 10mM, the cells failed to initiate growth in Tris-containing media. Results from this work indicate that E. coli B will grow in Tris-containing media with phosphate concentrations as low as 0.5mM if the cells are pre-adapted.

Glucose-Phosphate Limited Chemostat

The chemostat imposes a particular growth rate on the bacteria growing in it. The theory and operation of the chemostat used in these experiments has been adequately treated by Norris (1970) and Novick and Szilard (1950) and will not be developed here. However, the principle of the chemostat is to control the concentration of a limiting nutrilite of which the bacterial growth rate is dependent upon. Various factors such as flow rate, tube resistance, cultural volume, length of the tubing and hydrostatic pressure all play a major role in limiting the concentration of the nutrilite. However, all of the various measurements and conditions for the above factors were based on a glucose limited chemostat. Under these conditions, it was not known to this laboratory whether the Km for phosphate was higher than glucose or vice versa. Since a limiting concentration of phosphate was used (limiting at 0.5 at A_{600nm} for batch culture), it was of concern to us whether the chemostat under these conditions was still glucose-limited and not phosphate-limited. The results in Table 1 indicate that the chemostat under these conditions was glucose limited. Table 1 shows that the cell densities vary when the glucose concentration was varied and not when the phosphate concentration was varied.

Dry Weight Determinations

Norris (1970) determined dry weights turbidimetrically by referring the absorbance at 420nm (Cary model 16 Spectrophotometer) to a standard curve of absorbance versus dry weight. Bacteria growing at doubling

times from 1 to 24 hours gave identical standard curves up to an absorbance of 0.5 (Norris, 1970). Using Norris's conversion curve a similar standard curve was constructed at an absorbance of 600nm (Hitachi Perkin Elmer 139 U. V. - visible Spectrophotometer). Figure 2 shows the standard curve.

Sampling Cultures

Sampling and killing cultures was achieved by mixing the cell culture directly with weak acids. Strong acids (Perchloric acid) and (Trichloroacetic acid) were tedious to remove before chromatography and cause nucleotide degradation (Cashel, 1973). Aeration during sampling was never interrupted since artificial pool fluctuations may occur.

Extraction

One-hundred µl aliquots of culture were removed at designated times during each experiment and dispensed into tubes containing an equal volume of 4M sodium formate (pH 3) and kept in ice for 15-20 minutes. After extracting, much of the sample was transferred to microcentrifuge tubes (Beckman #314326) and cellular debris was centrifuged out at room temperature for 2 minutes in the Beckman microfuge (152 microfuge). The extracts were frozen at -25 C and thawed samples were resedimented prior to spotting. Extracts have been found to be stable for 2 weeks (Cashel, 1973).

Table 1. Bacterial growth patterns under limiting concentrations of glucose and phosphate.

KH_2PO_4[1]	Glucose[2] in g/100 ml	Absorbance[3] units in A_{600}
0.5	0.02	0.25
2	0.02	0.25
0.5	0.2	0.52

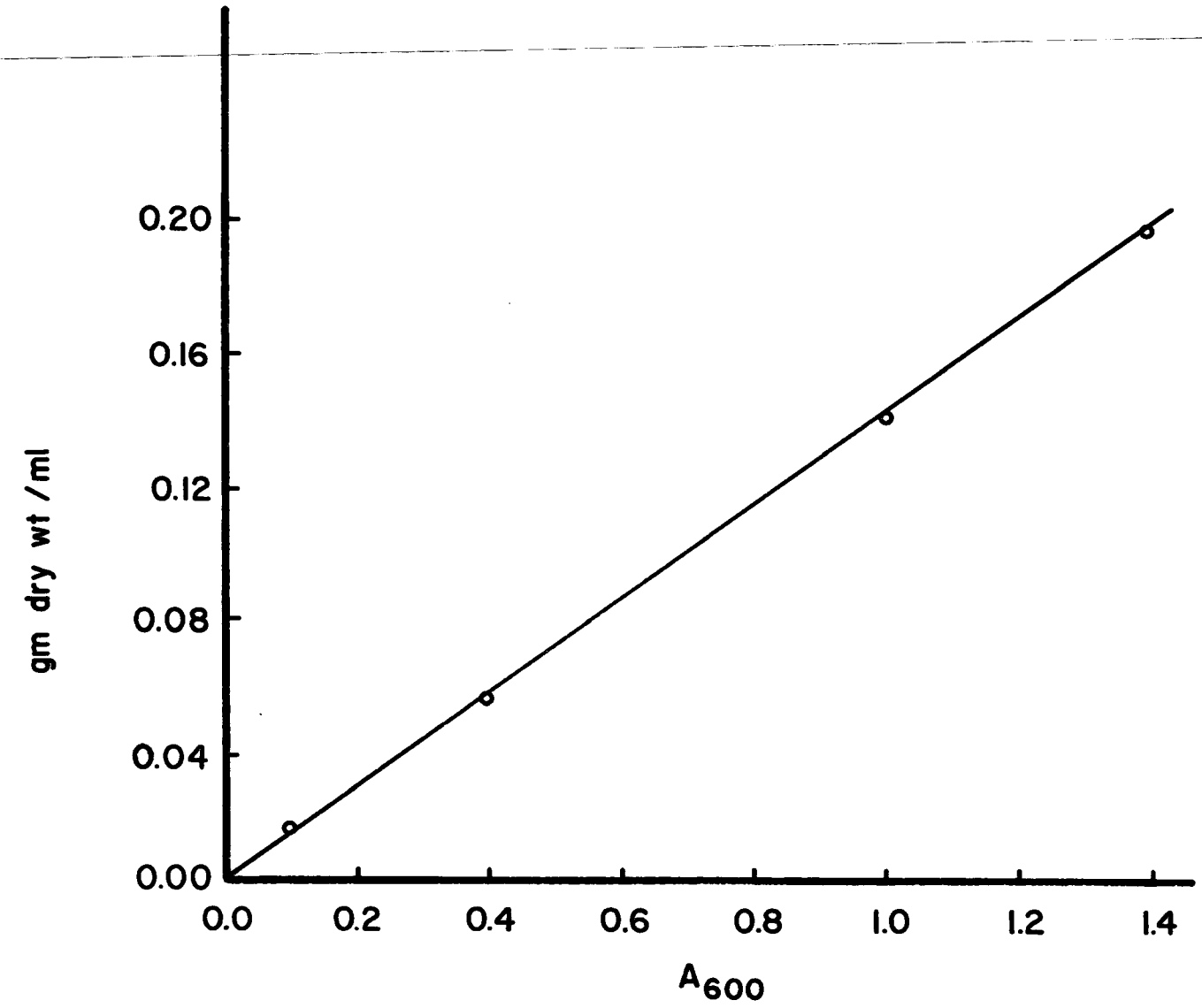
[1]The concentration of KH_2PO_4 is expressed in mm of solution.

[2]The concentration of glucose is expressed in gram percent (grams per 100 ml).

[3]The absorbance of the culture was read at 600 nanometers.

Fig. 2. Determination of the grams of dry weight of bacteria per mililiter of culture versus absorbance units at 600 nanometers.

A correlation between the grams of dry weight (gdw) per ml of E. coli B cells and the absorbance of the culture at 420nm was determined by Norris (1970). The relationship between the grams of dry weight per ml of culture and the absorbance at 600nm was determined by measuring the absorbance of E. coli B cells at 600nm and 420nm and reconstructing the curve at 600nm (shown in this figure). Comparison of this curve with the curve at 420nm (not shown) indicates that an absorbance at 600nm represents 80% of the dry weight that would be obtained if the absorbance were determined at 420nm.


0.20
0.16
0.12
0.08
0.04
0.00
gm dry wt /ml
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
A600

Chromatography

Plates.

Phosphoethyleneimine-impregnated-cellulose coated plastic plates without fluorescent indicator, available as Polygram cell 300 PEI, made by Machercy-Nagel and distributed by Brinkmann Instruments or Mathieson Company were used in all experiments. The coating on the plates was 0.1 mm thick. The plastic backed plates were used but were not necessary unless double-labeled experiments are conducted since tritium (^{3}H) and ^{14}C do not penetrate through the plastic back (Cashel, 1973). Prewashing of plates prior to use was necessary if they appeared yellowish in color. This is due to PEI decomposition. Finally, plates were written on with an electrographic pencil, even prior to chromatography.

Spotting.

A small amount (10-25 µl) of the extract was spotted directly on the chromatogram in one application using disposable micro-pipettes (made by Drummond Scientific Company and distributed by VWR Scientific, Inc.). Repeated spotting of smaller amounts was not necessary and even undesirable since it concentrates salts in a limited area (Cashel, 1973).

For one-dimensional chromatography, which was used for survey purposes, origin spots were established at 2.0 cm above one edge of the plate. The curled edge of the plate (the plate has a tendency to fold) was used so that the plate stands upright all alone and development proceeds as preferred, perpendicular to the direction of spreading.

For two-dimensional plates, 25 µl was spotted in one corner at the intersection of the origin lines. After spotting, all plates were dried for 3 minutes under a current of cold air.

Standards.

Nucleotide and nucleoside standards were stored at - 25 C as 10mM solutions. Ten µl of the solutions were spotted either for survey purposes to confirm the relative migration (RpHF) values or as ultraviolet (U. V.) markers to locate the area containing the radioactive component. However, 2 µl will give a dark U. V. spot. The predominant U. V. spot will generally correspond to the standard; contamination of 5' -triphosphates with 5' di- and 5' tetraphosphates is common (Cashel, 1973).

Containers for chromatographic development.

Relatively air tight, small glass containers were employed in all experiments. Glass boxes with 4-6 compartments and a glass lid were the most frequently used containers. Development in the first dimension takes 2-3 hours, while two-dimensional development takes 6-8 hours depending on the solvent system used. In all cases, the solvent was allowed to sit in the air tight container for at least 1 hour prior to use. The plates were stacked relatively close to each other but cellulose surfaces were not allowed to touch anything. Chromatography in open vessels give different relative migration values from those in closed containers (Randerath and Randerath, 1964).

Survey of developing solvents.

The choice among methods of development depends upon activities anticipated, the nucleotides to be resolved, and how carefully one

wishes to be in excluding minor cochromatographing contaminants. For survey purposes of all ribonucleotides, one-dimensional development in 0.75M KH_2PO_4(pH 3.4) was employed. The lower molecular weight nucleotides (mono and diphosphates) migrated further than the heavier molecular weight nucleotides (triphosphates). The degree of migration depends upon the net charges, size, shape, and molecular weight of the nucleotide. The RpHF values and the distance traveled by the nucleotide relative to the location of the pH front are constant. The pH front is located below the solvent front and observed only under U.V. light. RpHF values of each ribonucleotide as well as some deoxynucleotides were computed and the degree of resolution evaluated. Table 2 gives the RpHF values of all mono-, di-, and triphosphates.

For more careful resolution of purine mono-, di-, and triphosphates, one-dimensional development using step formate was employed. For step formate development, chromatograms were allowed to develop in 0.5M Na formate to origin, 2M Na formate to 2 cm above the origin and 4M formate to 15 cm above the origin. Table 3 gives an account of the RpHF values of purines and pyrimidines. There was co-migration of some purines with pyrimidine nucleotides by this method, but the method is useful when labelling with a free base or nucleoside of purine or pyrimidine nature. Thus, if one labels with a purine none of the pyrimidines will be radioactive.

To resolve the ribonucleoside triphosphates from the deoxyribonucleoside triphosphates, two-dimensional development in borate-phosphate was employed. In the borate-phosphate system, 3.3M ammonium formate + 4.2% boric acid (adjusted to pH 7 with NH_4OH) was used in the first

Table 2. Survey of the relative migration (RpHF) of nucleotides in 0.75M KH_2PO_4.

Nucleotides	RF[1] values	RpHF[2] values
GMP	0.568	0.929
GDP	0.401	0.657
GTP	0.228	0.374
TTP	0.564	0.894
UDP	0.630	1.000
UMP	0.630	1.000
UTP	0.576	0.930
AMP	0.581	0.891
ADP	0.529	0.812
ATP	0.271	0.416
TMP	0.660	1.000
CMP	0.654	0.990
CDP	0.638	0.996
CTP	0.455	0.689

[1]RF values = $\frac{\text{distance traveled by sample}}{\text{distance traveled by solvent front}}$

[2]RpHF values = $\frac{\text{distance traveled by sample}}{\text{distance traveled by pH front}}$

dimension while 0.75M KH_2PO_4 was used in the second dimension. Table 4 gives an account of the RpHF values of different triphosphates by this method. In all experiments correlating the size of the GTP pool with the growth rate of E. coli, this method of development was the one employed.

Two-dimensional development.

After developing in the first dimension, the plates were fan dried with a cold current of air. The chromatogram was examined under short-wave U. V. light and the section of the plate containing the pH front was cut off. The chromatogram was then soaked in a tray containing 350 ml of 85% methanol for 20 minutes to remove the developing solvent in the first dimension. This treatment removes approximately 10% of the nucleotides (Randerath and Randerath, 1964). The plate was then fan dried again and chromatography in the second dimension was carried out.

Quantitation

Autoradiography.

After development, the plates were dried in an oven (about 100 C). Then chromatograms were marked with radioactive ink spots (which does not wash off during autoradiography), to allow later realignment with film. The plates were then exposed to no-screen, medical X-ray film (Kodak) 8 x 10 inches. The film was held flat on the surface of the chromatogram with a 1/8 inch lead plate cut 20 x 20 cm. Four to five day exposures were allowed for tritium. Visible film darkening can be obtained with about 1000 cpm of tritium. After exposure, the film was developed with standard (Kodak) X-ray developer for 3 minutes,

Table 3. Survey of the relative migration (RpHF)[1] of nucleotides in step formate.[2]

Purines	RpHF values	Pyrmidines	RpHF values
GTP	.250	CTP	.523
ATP	.510	UTP	.410
GDP	.592	UDP	.629
ADP	.631	CDT	.858
GMP	.802	CMP	.110

[1] $\text{RpHF} = \frac{\text{distance traveled by the sample}}{\text{distance established by the pH front}}$

[2] Develop chromatogram in 0.5M Na formate (pH-3.4) to origin 2M Na formate to 2 cm above the origin and in 4M Na formate to 15 cm above the origin.

Table 4. Comparison of the relative migration of ribonucleotides and deoxyribonucleotides in borate-phosphate nucleotide triphosphate.

	$\frac{RpHF_2}{RpHF_1}$
GTP	$\frac{.228}{.251}$
dGTP	$\frac{.228}{.562}$
ATP	$\frac{.271}{.310}$
dATP	$\frac{.271}{.510}$

$RpHF^1$ = RpHF value in 1st dimension, where 3.3M ammonion formate + 4% boric acid is used as a solvent.

$RpHF^2$ = RpHF value in 2nd dimension, where 0.75M KH_2PO_4 is used.

rinsed with water once, and fixed with standard (Kodak) X-ray fixer for about 5 to 15 minutes or until clear. The film was then washed for 20 minutes in running water, and dried in an oven. The film was then superimposed on the chromatogram and aligned with ink marks. Activity spots were delimited on the chromatogram by pricking a hole through the film with a dissecting needle. The appropriate areas of the chromatogram (as well as a blank) were cut out and counted by liquid scintillation in Aquasol (New England Nuclear).

Efficiency of counting Tritium on PEI-cellulose.

The efficiency of counting tritium was determined in the following manner. First, an aliquot of culture containing (8-3H) guanine (specific activity 12 Ci/mmole) was spotted on a PEI-cellulose chromatogram. The spot was cut out, placed in a vial containing 10 ml of scintillation fluid and counted by liquid scintillation. Secondly, to the above sample, an internal standard with a known number of disintegrations per minute (dpms) was added and recounted.

The efficiency of counting tritium was calculated by subtracting the number of counts per minute (cpm) in the sample alone from the number of cpm in the sample plus the internal standard. This value (in cpm) was divided by the known number of dpms of the internal standard to yield 15.6 as the efficiency factor.

Sensitivity and reproducibility of the method.

The sensitivity of the method using radioactivity was determined by expressing radioactive samples per gram of dry weight of cells used in the assay. Table 5 gives an account of this sensitivity.

Table 5. The reproducibility and sensitivity of the method.

A. Reproducibility

Time[a] (sec)	cpm/12.5 µl	
	Exp 1[b]	Exp 2[c]
	GTP	
20	625	656
40	714	722
240	2710	2796
	ATP	
20	97	118
40	158	148
240	506	515

B. Sensitivity

µ moles GTP[d] gram dry wt.	µ µ moles GTP[e] 12.5 ul
0.10	.075

[a]refers to the incorporation time allowed for (8-3H)guanine to enter the GTP or ATP pools.

[b]counts per minute (cpm) were obtained by spotting a 25 µl sample composed of equal volumes of 4 M Na formate and radioactive culture onto PEI-cellulose. The chromatogram was developed and ATP and GTP isolated and counted.

[c]counts per minute were obtained using the identical procedure described above for b at a time interval 7 days later.

[d]the pool size was assumed to be 1 µ mole/gdw based on data by Wong and Nazar (1972). The radioactivity in the GTP pool 1.4 minutes after the addition of radioactive guanine was assumed to represent 10% of saturation. At this time the absorbance of the culture was 0.4 units at A_{600}. (These assumptions were later shown to be valid as shown in Figure 4).

[e]each ml of cells had 0.056 mg dry wt/ml. 12.5 µl was assayed to give 0.75 µg dry wt/12.5 µl.

The reproducibility of the method was determined by developing chromatograms in phosphate-borate (two-dimensional) solutions on two separate occasions 1 week apart. Table 5 also gives an account of these results.

Short Term Incorporation of (8-3H)guanine into GTP Pool of E. coli B Cells Doubling Every 1.5 Hours

Overnight cultures of E. coli B, growing in Tris-glucose minimal media supplemented with 0.2% glucose, IX minerals and 0.5 mM KH_2PO_4, were diluted 10-fold into fresh media containing the same supplements. The culture was incubated at 37 C under forced aeration. The absorbance of the culture was periodically determined and the mg of dry weight per ml versus time was graphed on semilogarithimic paper. When the culture reached an absorbance of 0.4 at A_{600nm} corresponding to a concentration of 0.056 mg of dry weight per ml and after the cells had gone through 2 doublings times (doubling time was 90 minutes), 4 ml of the cells were dispersed into a tube containing 0.2 ml of (8-3H) guanine (specific activity 12 Ci/mmole; conc. 0.5 mCi/ml). Samples were then taken at 20 second intervals for 4 minutes by removing 0.1 ml of culture with 100 µl Eppendorf pipettes. The samples were pipetted into tubes containing 4 M formic acid (pH 3) and extracted on ice for 20 minutes. To determine the total counts in and on the bacteria, 0.1 ml samples were collected into 10 ml of aquasol and counted in the liquid scintillation counter. In addition, 0.1 ml samples were taken from a non-radioactive control culture and treated by the same procedures.

Extracts were then centrifuged in the Beckman microfuge for 2 minutes at room temperature, after which two-dimensional chromatography on PEI-cellulose was conducted using borate-phosphate development. Twenty-five μl of the extract was spotted at the intersecting origins along with 10 μl of standard 10 mM non-radioactive GTP and ATP. After development, GTP and ATP spots were located under short U.V. light while the relative location of dATP and dGTP were estimated by their RpHF values in both dimensions. This latter procedure proved to be convincing since all chromatograms were developed 15 cm above the origin in both dimensions. In addition, autoradiographic procedures were employed for some chromatograms to further validate the location of the radioactive ribo- and deoxyribo-nucleoside triphosphates relative to U.V. markers and RpHF values. Spots containing GTP, dGTP, ATP, and dATP were counted and the counts per minute (cpm) normalized per gram dry weight of cells using the growth curve shown in Figure 2.

Long Term Incorporation of (8-3H)guanine into GTP Pool of *E. coli* Cells Doubling Every 1.5 Hours

The methods, materials and bacterial strain were the same as in the short term experiment. However, the guanine concentration was increased to make the final concentration of exogenous guanine in the medium 10.93 μM. The concentration was increased with non-radioactive guanine such that the specific activity of the radioactive guanine changed from 12 Ci/mmole to 2.2 Ci/mmole. When the cells reached an absorbance of 0.35, 4 ml of cells were added to 0.6 ml of (8-3H) guanine (specific activity, 2.2 Ci/mmole) and 100 μl samples were taken

at 2 and 3 minute intervals for 35 minutes at which time the absorbance of the culture was at 0.4.

Ribo- and deoxyribo-nucleoside triphosphates were extracted, isolated, counted and corrected by the same procedures described in the previous section.

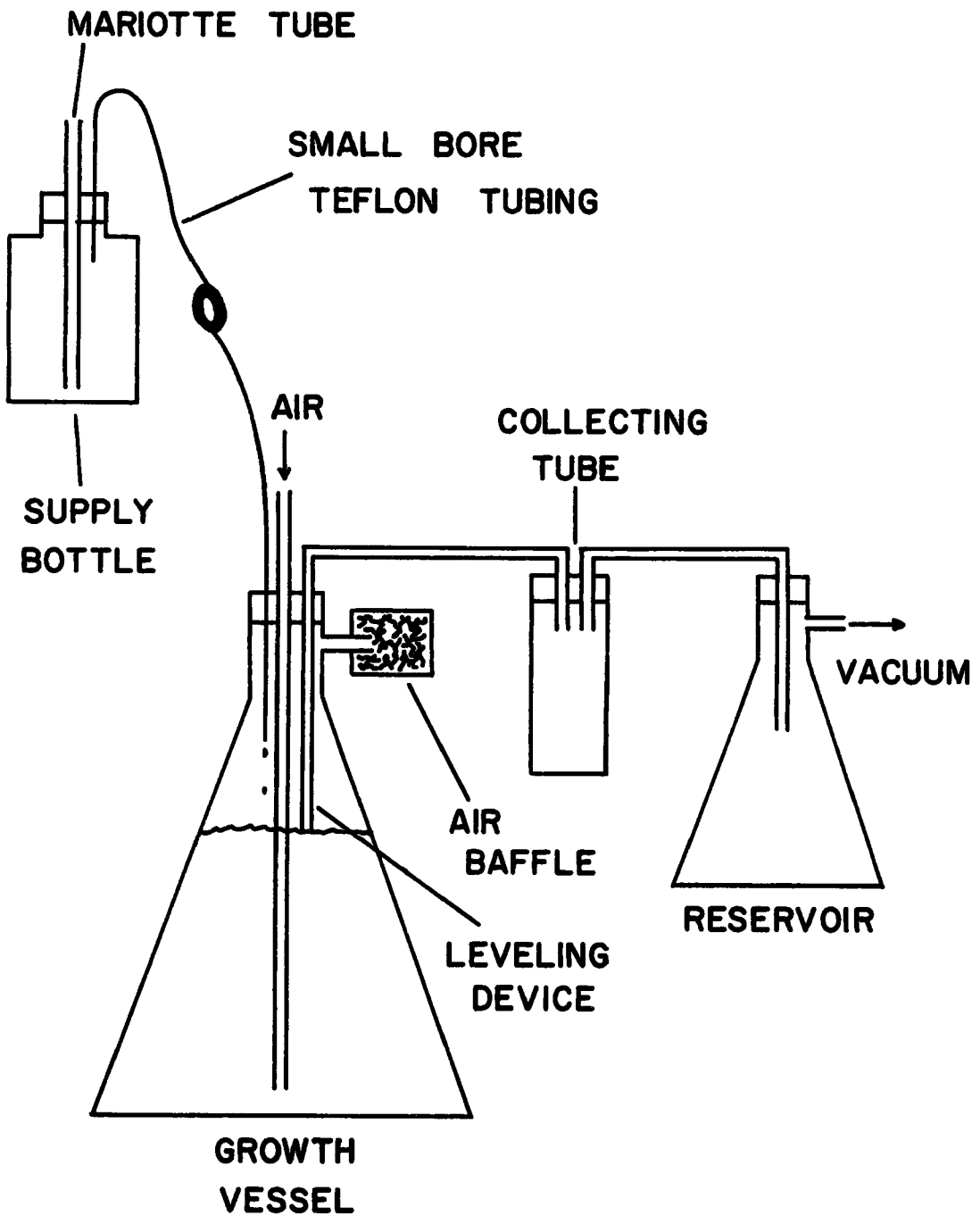
Short Term and Long Term Incorporation of (8-3H)guanine into GTP Pool of Chemostat Cells Doubling Every Ten Hours

The theory, description and operation of the present chemostat is described by Norris (1970) and will not be discussed in detail in this paper.

A 1000 ml chemostat was set into operation by adjusting the flow rate at which the media is supplied to a value of 69.3 ml/hour. The flow rate is governed by the hydrostatic pressure and the resistance in the teflon tubing which delivers the media from the supply bottle to the growth vessel. The hydrostatic pressure is adjusted by increasing or decreasing the distance between the bottom of the supply bottle and the top of the growth vessel where the teflon tubing ends (See Figure 3). The resistance in the tubing is proportional to the length of the tubing. Thus, the doubling time (T_2) of the culture is expressed as 0.693 x 1000 ml/69.3 ml/hr where 0.693 is the natural logarithm of 2, 1000 ml is the volume of cells in the growth chamber in ml per hour. The doubling time (T_2) under these conditions is equal to 10 hours. The desired flow rate, 69.3 ml/hr, was obtained by using 10 feet of teflon tubing and adjusting the hydrostatic pressure to a height of 58.2 cm. Air was delivered to the growth chamber, to provide adequate aeration

Fig. 3. A schematic diagram of the present chemostat.

The Mariotte supply bottle delivers media through the teflon resistance siphon tubing to the growth chamber which is maintained at a constant temperature in a water bath. Flow to the growth chamber is controlled by the hydrostatic head (distance between the bottom of the Mariotte tube and the end of the siphon) and by the resistance offered by the siphon tubing (proportional to the length of the tubing). Media and bacteria are removed from the growth chamber by a constant leveling device connected to a vacuum source. The leveling device is bent away from the point of entry of new media in order to minimize the possibility of removing freshly added medium before it becomes mixed with the entire growth media. A collection vessel attaches to the top end of the constant leveling device. Bacteria are removed to the collection device through a 60 cm length of teflon tubing into the glass tube. The vacuum side of the collection vessel is connected to a 4 liter filter flask which acts as an overflow reservoir and is in turn connected to a vacuum pump.


MARIOTTE TUBE
SMALL BORE
TEFLON TUBING
AIR
COLLECTING
TUBE
SUPPLY
BOTTLE
VACUUM
AIR
BAFFLE
RESERVOIR
LEVELING
DEVICE
GROWTH
VESSEL

and mixing through a sterile air filter which attaches to a water saturated laboratory supply (maintained at 5 pounds/square inch by a pressure regulator not known in Figure 3) at one end and to a glass sparger (200 ml units) at the other end. To a sterile supply bottle and growth chamber, containing the Tris-glucose media are added 0.02% glucose, IX minerals and 0.5 mM KH_2PO_4. An overnight culture of cells growing in Tris-glucose media supplemented with 0.2% glucose and 0.5 mM KH_2PO_4 was diluted tenfold into a volume of 900 ml of media contained in the growth chamber. Flow was started by attaching an air supply to the top end of the Mariotte tube (located on the supply bottle) thereby forcing media through the resistance siphon tubing into the growth flask. When flow was started, the pressure in the supply bottle was released by loosening the Mariotte tube stopper which does not cause contamination of the supply bottle. Media and bacteria were removed from the growth chamber by a constant leveling device (Figure 3) connected to a vacuum source.

The growth chamber was maintained at a temperature of 37 C. Cells were allowed to proceed through three generations to reach steady state and the absorbance of the culture was determined periodically.

Because large amounts of radioactivity were required to experiment with the 1000 ml chemostat, a smaller 25 ml chemostat was constructed. Twenty-five ml of cells were transferred from the 1000 ml volume chemostat into a smaller 25 ml chemostat. The smaller chemostat was set up in the following manner: The resistance in the tubing was doubled by increasing the length of the tubing from 10 feet to 20 feet; the hydrostatic pressure was adjusted to a height of 1 cm; and the volume of the

growth chamber was maintained at 25 ml. The flow rate was adjusted to 1.7 ml/hr, giving the culture a doubling time of 10 hours. This can be seen in the following equation where T_2 equals 0.693 x 25 ml/ 1.72 ml/hr. The small 25 ml chemostat was allowed to reach steady state by allowing the culture to double through 6 generations.

However, during the experiment media and bacteria were not removed from the growth chamber by the constant leveling device. This procedure was done so that none of the radioactivity was lost from the growth chamber. Rather the data were corrected for the increase in absorbance. Furthermore, the flow rate during the experiment was controlled manually by dispersing 28 µl of media plus supplements into the growth chamber every minute. Prior to beginning the experiment the (8-3H)guanine (specific activity 2.2 Ci/mmole) was prewarmed in the 37 degrees C water bath.

At time zero, 2.1 ml of (8-3H)guanine (giving final guanine concentration of 6.14 µM) was dispersed into the 25 ml chemostat. Then 100 µl samples were taken at 20 second intervals for 4 minutes to assay short term incorporation, 2 and 3 minute intervals for 56 minutes and at 30 minute intervals for 1 hour to assay long term incorporation. The amount of GTP and ATP was assayed as described in the previous section.

CHAPTER IV

EXPERIMENTAL RESULTS

Incorporation of Radioactive Guanine into GTP and ATP Pools of Cells Doubling Every 1.5 Hours

It was of intent to determine the steady state size of the GTP pool of E. coli cells doubling every 1.5 hours and to determine the time required for equilibration of radioactivity in the pool when labelled under steady state conditions.

Figure 4 presents the results of an experiment designed to answer this question. Radioactive guanine was allowed to incorporate for 37 minutes into the GTP and ATP pools of E. coli. The curves are constructed to express the total amount of radioactivity in and the specific activity of GTP as a function of time. The left-hand ordinate is expressed in disintegrations per minute (dpms) per ml and per absorbance at 600nm. The right hand ordinate is an expression of specific activity given in terms of curies (Ci) per mmole. It should be pointed out that the values in the right ordinate apply only to the GTP pool and not the ATP pool.

Figure 4 shows a rapid exponential rise in the GTP pool (denoted by the symbol o) for approximately 13 minutes. The curve then bends and becomes linear at 17.3 minutes and continues this pattern for the next 8-9 minutes. After 24 minutes of incorporation there appears to be a uniform but slower decline in the pool over the next 13 minutes.

Figure 4 also shows that the pool saturates at approximately 1,000,000 dpms which corresponds to a specific activity of 2.2 Ci/mmole as shown at the right hand ordinate.

This value for the specific activity at that point in time is based on the assumption that the specific activity of the GTP pool equals the specific activity of the exogenous source when the pool is fully saturated. The specific activity as a function of time is then extrapolated back to zero time to give the values for the specific activities observed in Figure 4.

The ATP pool (designated by the symbol x) which incorporates radioactivity under these conditions shows a similar pattern as the curve shown for GTP. The saturation time, however, cannot be determined since de novo synthesis of adenine is not shut off. Thus the specific activities of the ATP pool as shown in Figure 4 cannot give a clear indication of its size. It should be noted at this point that the amount of radioactivity incorporated into the ATP pool is 3.3-fold less than the amount incorporated into the GTP pool. Figure 4 also points out that the rate of decline of the pool (both ATP and GTP) is slower than the rate of uptake.

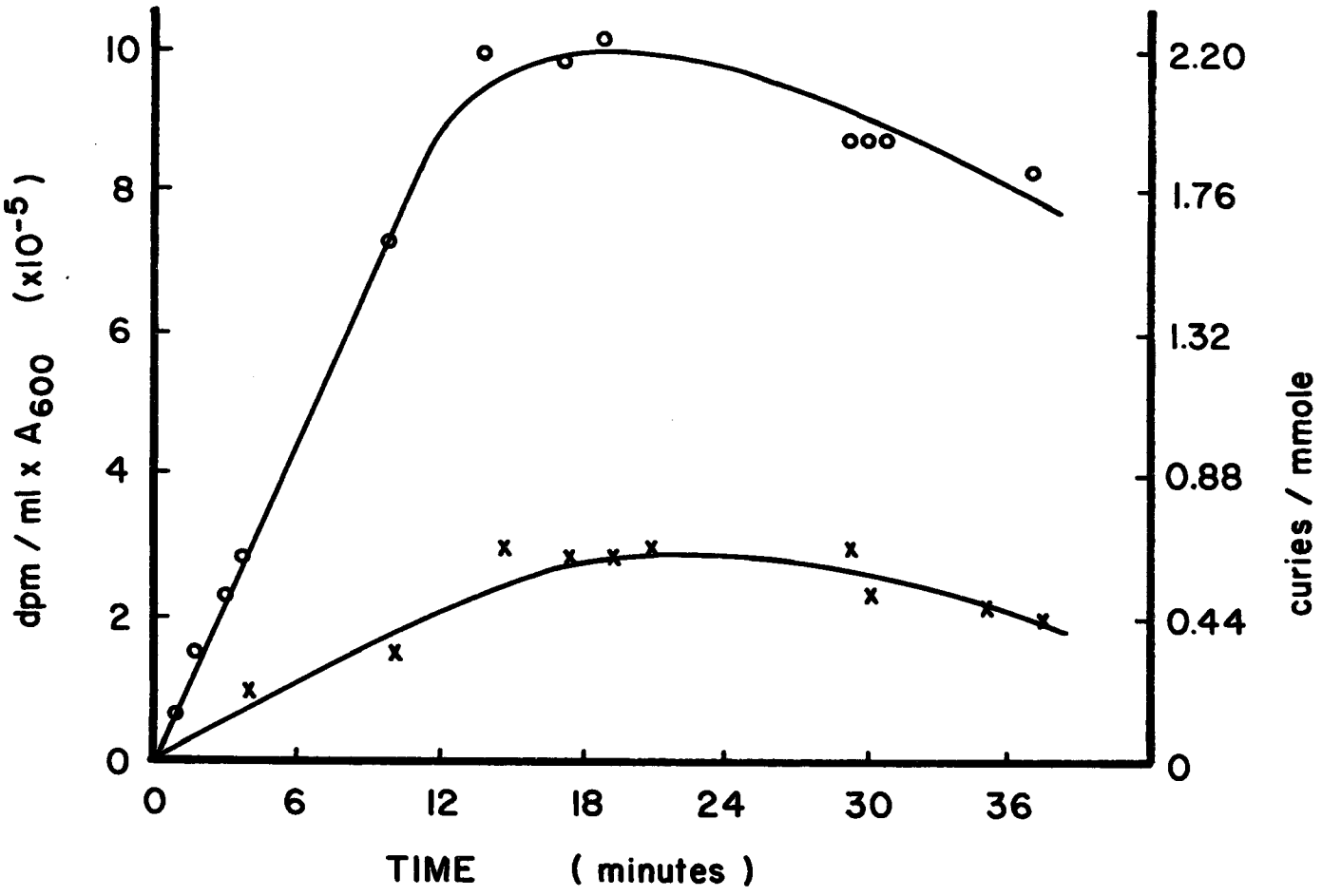
Incorporation of Radioactive Guanine into GTP and ATP Pools of Cells Doubling at 10 Hours

The following experiment was done to find out the steady state size and the time required for equilibration of radioactivity within the cellular GTP pool of *E. coli* cells doubling every 10 hours. Figure 5 presents the results of this experiment in which (8-3H)guanine was allowed to incorporate into the GTP and ATP pools of slow-growing

Fig. 4. Incorporation of guanine into GTP and ATP pool of cells every 1.5 hours.

Four ml of an exponentially growing culture of E. coli cells growing in a low phosphate Tris-glucose minimal media (See Material and Methods) was transferred to a prewarmed container with 0.6 ml of radioactive guanine (specific activity 2.2 Ci/m mole). Two tenths of a ml of radioactive guanine (specific activity 12 Ci/m mole, concentration 1.63 μM) was diluted with 0.4 ml of non-radioactive guanine (concentration 95.5 μM) to give a final guanine concentration within the culture of 10.93 μM. The concentration of non-radioactive guanine was assayed spectrophotometrically at an absorbance of 248 nm. The culture was treated as described in Material and Methods and samples counted with 15.6% efficiency. The data is expressed as dpm per ml of culture per absorbance at 600 nm or as the specific activity of the pool in Ci/m mole.

The symbol o-o represents data points for GTP while the symbol x=x represents data points for ATP.


10
8
6
4
2
0
dpm / ml x A600 (x10-5)
2.20
1.76
1.32
0.88
0.44
0
curies / mmole
0
6
12
18
24
30
36
TIME (minutes)

chemostat cells for 140 minutes. The symbols for the ATP and GTP curve are the same as those described in the previous section of the results.

The results in Figure 5 show a rapid rise in the GTP pool over the first 2 minutes. Then there is a slower and nearly linear rise over the next 50 minutes. The curve appears to plateau near 66 minutes and remains linear for the next 19 minutes. This saturation period is followed by a slow decline in the GTP pool for the next 55 minutes. The rate of decline in the GTP pool is also slower than the rate of uptake of radioactivity. The GTP pool saturates at 576,000 cpm which corresponds to a specific activity of 2.2 Ci/mmole as shown in Figure 5. The specific activity at that point was extrapolated back to zero as described in the previous section of the results.

Figure 5 also shows that the ATP pool fills up nearly identical to that of the GTP pool. A curve was not drawn to show the relationship to its data points to avoid confusion in interpreting the graph. However, the pattern of the curve can be seen by observing the x's (data points) on the graph shown in Figure 5. As previously pointed out, the right ordinate in Figure 5 expresses the specific activity of the GTP pool and cannot be applied to the ATP curve.

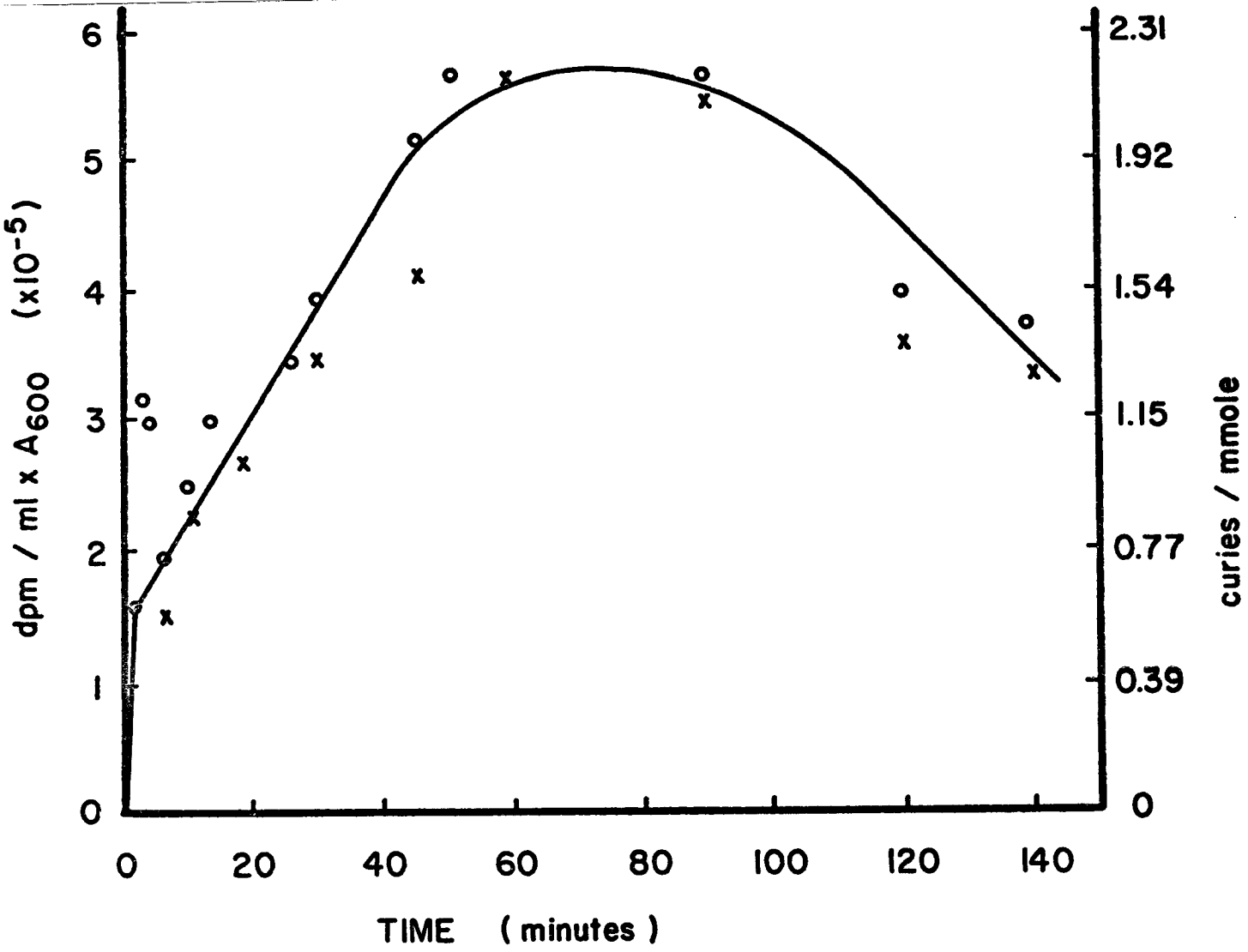
Short Term Incorporation of Radioactive Guanine into GTP pool of Cultures Doubling at 1.5 Hours

To find the specific activity of the GTP pool at very short labelling times, the experiment represented by Figure 6 was performed. Radioactive guanine was allowed to incorporate for 240 seconds into the GTP pool of *E. coli* cells doubling every 1.5 hours. Figure 6 shows an initial rapid rise extending over the course of the first 28

Fig. 5. The incorporation of H^3 guanine into GTP and ATP pool of cells doubling every 10 hours.

Twenty-five ml of chemostat cells were transferred to a container which had 1.42 ml of 95.5 µm non-radioactive guanine and 0.71 ml of H^3 guanine (specific activity: 12 Ci/mmole; concentration .5 mCi/ml). The radioactive guanine was diluted to a final specific activity of 2.2 Ci/mmole while the final guanine concentration within the culture was 6.14 µm. Samples were counted at 15.6% efficiency. The chemostat culture was treated as described in Material and Methods and the data expressed in terms of dpm per ml of culture at an absorbance of 600nm or as the specific activity of the pool in curies per mmole.

The symbol o-o represents data points for the GTP pool while the symbol x-x represents data points for the ATP pool.


dpm / ml x A600 (x10-5)
curies / mmole
TIME (minutes)
0
1
2
3
4
5
6
0.39
0.77
1.15
1.54
1.92
2.31
0
20
40
60
80
100
120
140

seconds into the GTP pool of E. coli cells doubling every 1.5 hours. Figure 6 shows an initial rapid rise extending over the course of the first 28 seconds and is followed by a slower rise which appears rapid over a short labelling time of 4 minutes. The data shown on the left hand ordinate of Figure 6 were determined experimentally (with corrections as described in the legend or summary table) and expressed as dpm per ml and per A_{600}. The right ordinate expresses the specific activity of the GTP pool in terms of mCi/mmole. In order to express the results of this experiment in terms of specific activity, it was necessary to match this short term data with the results shown in Figure 4 (Figure 4 did not resolve short time sampling periods but did indicate the saturation of the pool). To match the data from these two experiments (Figures 4 and 6) a point in time (4 minutes) was taken which was common to each experiment. This point was extrapolated back to zero to give the values for the specific activities shown in Figure 6.

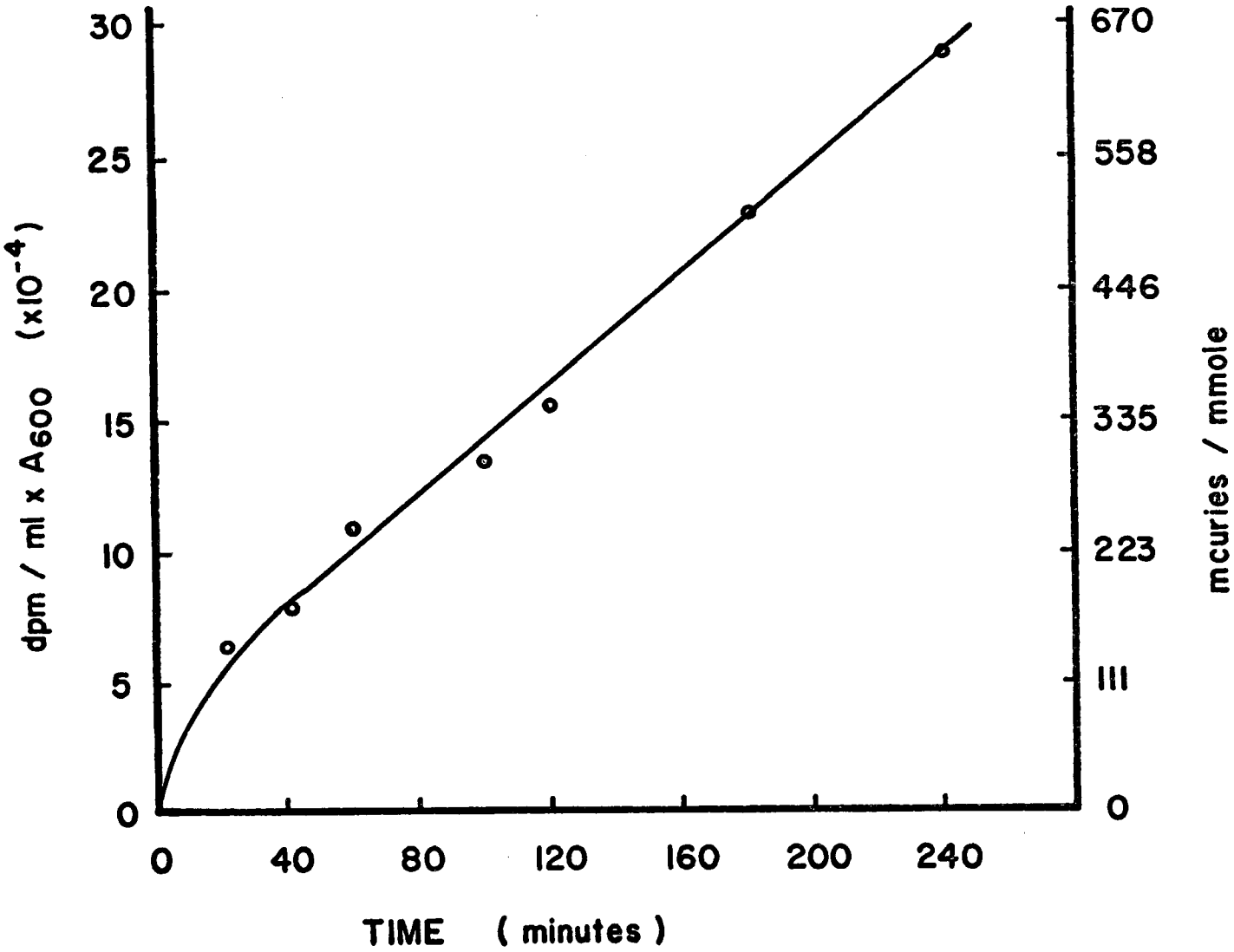
Short Term Incorporation of Radioactive Guanine into GTP Pool of Cells Doubling Every 10 Hours

The following experiment was conducted to find out the specific activity of the GTP pool over a short labelling period for E. coli cells doubling every 10 hours. Figure 7 shows the results of this experiment in which radioactive guanine was allowed to incorporate into the GTP pool for 240 seconds.

Figure 7 shows a very rapid but uniform rise for 120 seconds and a very slow but still rising uptake of radioactivity which appears linear over a short labelling time. The data are expressed

Fig. 6. Short term incorporation of guanine into GTP pool of cells doubling every 1.5 hours.

Five and one-tenth ml of exponentially growing cells of E. coli B were transferred to a prewarmed container of radioactive guanine (specific activity 12 Ci/mmole; concentration .5 mCi/ml). The final guanine concentration was 1.63 μM as 0.2 ml of the radioactive source was added to the culture. The culture was treated as described in Material and Methods and samples were counted at 15.6% efficiency. The short term and long term incorporation of guanine into the GTP pool was allowed to overlap so that the specific activity of 12 Ci/mmole could be expressed as 2.2 Ci/mmole. The data are expressed as dpm per ml per A_{600} and the specific activity corrected to 2.2 Ci/mmole. The specific activity of the pool is expressed as mcuries/mmole and was extrapolated using the long term incorporation results (Figure 4).


30
25
20
15
10
5
0
dpm / ml x A600 (x10-4)
670
558
446
335
223
111
0
mcuries / mmole
0
40
80
120
160
200
240
TIME (minutes)

in dpm per ml per A_{600} at the left ordinate and in mCi/mmole (specific activity) at the right ordinate.

The correlation between specific activity and time is made by using the same procedure as described in the previous section of the results. However, the specific activity of the pool at 4 minutes was determined from the curve shown in Figure 5. It can be noted that the specific activity of the GTP pool for a culture doubling every 1.5 hours (Figure 4) is the same as the specific activity for a culture doubling every 10 hours (Figure 5) at 240 seconds, but the amount of radioactivity incorporated (dpm per ml·A_{600}) differs greatly.

Summary of Results

Table 6 shows the results by giving the GTP pool sizes and the correction data used to calculate these values.

Fig. 7. Short term incorporation of guanine into GTP pool of cells doubling every 10 hours.

The same conditions and procedures described in the legend to Figure 5 were used for short term incorporation. The data are expressed as dpm per ml per A_{600}. The specific activity is expressed by the same procedure described in the legend to Figure 6.

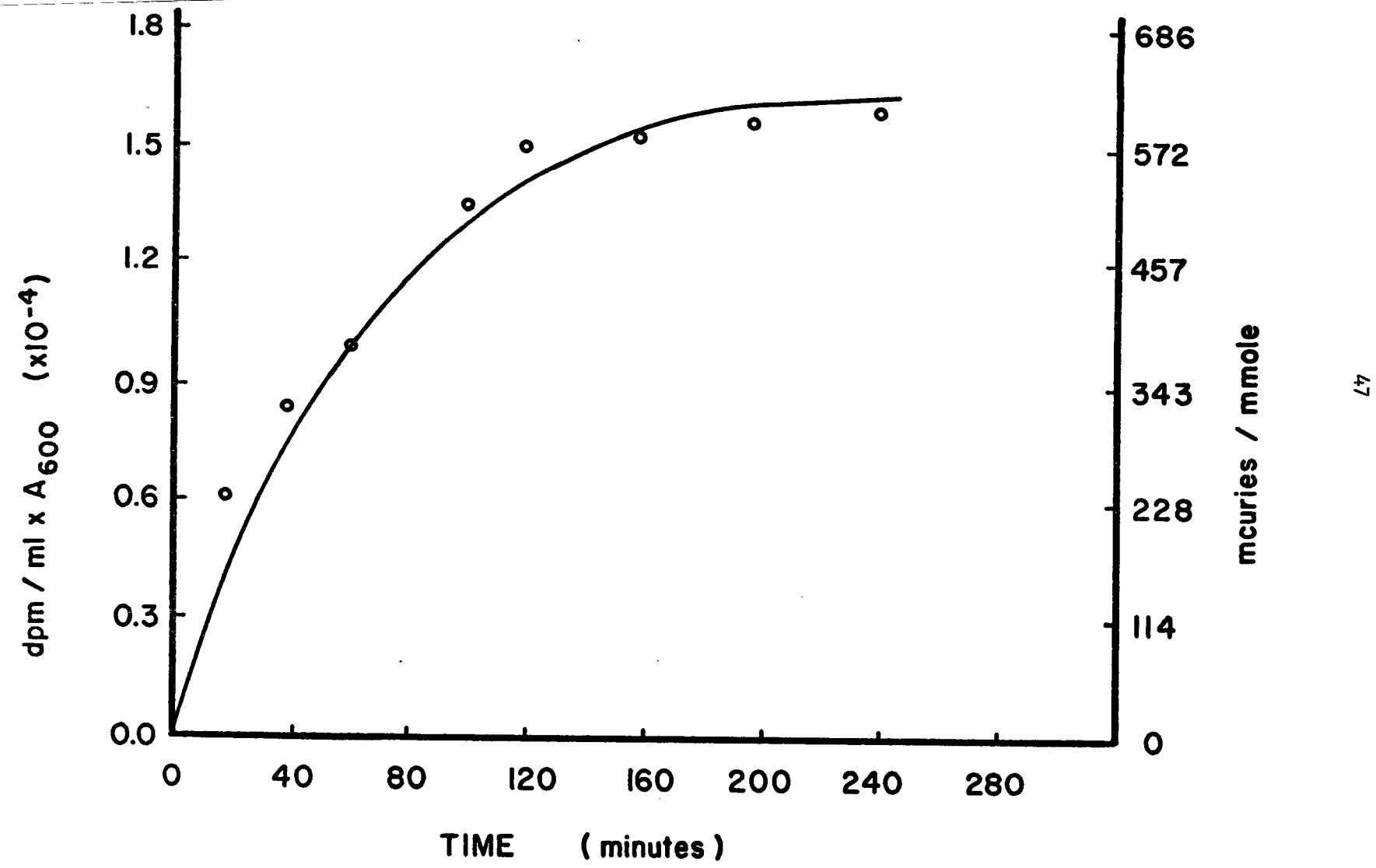

1.8
1.5
1.2
0.9
0.6
0.3
0.0
dpm / ml x A_{600} (x10^{-4})
0
40
80
120
160
200
240
280
TIME (minutes)
686
572
457
343
228
114
0
mcuries / mmole

Table 6. Summary table.

Time[a] (min)	T_2[b] (Hours)	$\frac{cpm^c}{ml \cdot A_{600}}$	$\frac{dpm^d}{ml \cdot A_{600}}$	$\frac{m\,\mu\,moles^e}{ml \cdot A_{600}}$	$\frac{\mu\,moles^f}{gram\ dry\ weight}$
17.3	1.5	156,000	1,000,000	1.4625	1.49
66.0	10	90,000	576,923	.11812	.8437

[a]is expressed in minutes and refers to the exact time the guanosine triphosphate pool becomes saturated.

[b]refers to the doubling time of the culture.

[c]cpm were corrected for growth to A_{600} and expressed in terms of ml volume.

[d]conversion of cpm to dpm is achieved by dividing by the efficiency, $15.6 \cdot \frac{dpm}{cpm}$.

[e]conversion to m μ moles/ml is achieved by converting the number of dpm/ml to μcuries/ml, (2.22×10^6 dpm = 1 μCi), and converting the number of μCi/ml into m μ moles/ml using the specific activity (2.2 Ci/m mole).

[f]conversion of m μ moles/ml . A_{600} to μ moles/gram dry wt is achieved using Norris's conversion curve, where an optical density of 1 at Abs. of 600 nm correspond to .14 mg of dry weight/ml.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The major point of this paper is that there is a correlation between the guanosine triphosphate pool levels and the total rate of RNA synthesis. Over the growth rate span studied, which is large in comparison to work from other laboratories, the sizes of the GTP pools do not show a vast degree of difference. It is also then, the object of this paper to show that such a large degree of difference in the GTP pools over the growth rate span studied is not needed to implicate the GTP pool size in a regulatory device.

A basic assumption made in interpreting the above data is that the GTP extracted from the cells is representative of the GTP used for RNA synthesis. In other words it is assumed that there is no significant compartmentalization or channeling within the pools of guanine ribonucleotides. Although it has been claimed that such compartmentalization exists, (Buchwald and Britten, 1953), more recent studies have proved the earlier results to be in error (Nierlich and Vielmetter, 1968).

This work indicates that the size of the GTP pool for cells of E. coli B doubling every 1.5 hours in steady state is 1.46 μmoles per gram of dry weight of bacteria. For chemostat cells of E. coli growing also in steady state and at a doubling time of 10 hours, this work indicates that the size of the GTP pool is 0.843 μmoles per gram of dry weight of bacteria.

Thus, according to these results there is a 1.66-fold reduction in the size of the GTP pool as the growth rate of the bacteria is slowed by a factor of 6.6. Also the average rate of uptake of radioactivity into the GTP pool is 6.6-fold slower for bacteria doubling every 10 hours as compared to bacteria doubling every 1.5 hours.

Norris and Koch (1972) using techniques of RNA-DNA hybridization estimated that the total rate of RNA synthesis per unit amount of DNA is twice as fast for cultures doubling every hour as it is for cultures doubling every 10 hours. However, their calculations involved the assumption that all messenger RNA molecules are synthesized at the same rate as the messenger RNA for beta-galactosidase. With the use of the data from Norris and Koch, this author has estimated that the total rate of RNA synthesis per unit amount of DNA is 1.7-fold greater for cultures doubling every 1.5 hours as for cultures doubling every 10 hours. Thus, since the size of the GTP pool is 1.66 times greater over the same growth rate span, it clearly follows that there is a correlation between the GTP pool and the total rate of RNA synthesis.

As shown in Chapter II of this thesis, there is another independent method of estimating the total rate of RNA synthesis. This method was developed by Bremer and Mueller (1968) and the equations appear on pages 11 through 15.

It was possible to use this equation because this work established the change in the specific activity, S, of the GTP pools of E. coli growing with doubling times of 1.5 and 10 hours over very short times after the addition of exogenous 8-3H guanine (Figures 6 and 7 respectively). The rate of uptake of radioactive guanine into the RNA of E. coli B

were taken from Bremer and Mueller (1968). Consequently, it was possible to calculate $V_2 + V_4$, the total rate of RNA synthesis for cells doubling every 1.5 hours on the one hand and for cells doubling every 10 hours on the other. This calculation indicates that there is a 2.0-fold reduction in the total rate of RNA synthesis as the growth rate is reduced over this growth rate span.

It follows then, that the rate of total RNA synthesis calculated above for cultures doubling every 2 hours with respect to cultures doubling every 10 hours is consistent with the 1.66-fold difference in the GTP pool of cultures doubling every 1.5 hours and 10 hours.

Furthermore, the 2.0-fold difference in the total rate of RNA synthesis which is calculated with the use of Bremer and Mueller's equation is consistent with the value obtained by Norris and Koch (1972). Since these results correlate this offers further support to the fact that all messenger molecules have the same average life span under the conditions described by Norris and Koch (1972).

The idea that ribosome synthesis might be controlled in such a way that rRNA would be synthesized in excess and then degraded was proposed by Rosset et al (1966) and documented by them (Julien, Rosset and Monier, 1968) for the case of phosphate starved cells. Control at the level of degradation was also proposed by Ehrenfeld and Koch (1968) whose work suggested that it might not be a trivial, but a normal biological control mechanism functioning under natural conditions. Norris and Koch (1972) showed that r- and tRNA does turn over. These concepts are also very strongly supported by data here.

Koch and Norris (1973) calculated that the rate of accumulation of RNA per unit amount of DNA was 144 times faster for a culture with a doubling time of 0.5 hours than a culture with a doubling time of 24 hours. Using Koch and Norris's equations, this author calculates that the rate of accumulation of RNA per unit amount of DNA is 13.4 times faster for a culture doubling every 1.5 hours than for a culture doubling every 10 hours. However, the results show that the total rate of RNA synthesis is only 1.7 times faster for cultures growing over the same growth rate span.

Thus, the rate of synthesis for slow-growing cultures is much faster than the rate of its accumulation. This is consistent with the results obtained by Norris and Koch (1972). It follows that ribosomal and transfer RNA must be unstable for slow-growing cultures and that a significant control on the amount of RNA in E. coli is at the level of degradation.

Furthermore, the mechanisms which regulate RNA synthesis do act in the selection of genes which are transcribed. Bremer et al (1973) showed that the fraction of RNA polymerases alloted for the synthesis of ribosomal RNA decreased as the growth rate was slowed. However, the growth span used by Bremer and Dennis (1973) was not as large of a growth span as the one employed in this investigation. In terms of future research, it would be interesting to observe whether the fraction of RNA polymerases for ribosomal RNA synthesis decrease slightly over a larger span of growth rates. It would be equally interesting to find out whether these RNA polymerase molecules are indeed being transferred from one RNA species to another as the growth rate is varied.

CHAPTER VI

SUMMARY

In summary, the data in this investigation support the following concepts: (1) The size of the guanosine triphosphate pool is correlated with the total rate of RNA synthesis and thus may be one of the control mechanisms affecting RNA synthesis. (2) Ribosomal and transfer RNA are unstable for slow-growing cultures. (3) Beta-galactosidase can possibly be considered an average messenger molecule under the condition established by Norris and Koch (1972). (4) A large degree of difference is not needed to correlate any nucleotide pool size with the total rate of RNA synthesis.

LITERATURE CITED

Bagnara, A. S., and L. R. Finch. 1968. Ribonucleoside triphosphate accumulation on amino acid starvation of "stringent" Escherichia coli. Biochem. Biophys. Res. Com. 33:15-21.

Borek, E., A. Ryan, and J. Rockenbach. 1955. Nucleic acid metabolism in relation to lysogenic phenomenon. J. Bact. 69:460-467.

Bremer, H., L. Berry, and P. Dennis. 1973. Regulations of ribonucleic acid synthesis in Escherichia coli B/r: An analysis of a shift-up. II. Fraction of RNA polymerase engaged in the synthes of stable RNA at different steady state growth rates. J. Mol. Biol. 75:161-179.

_____and P. Dennis. 1973. Regulation of ribonucleic acid synthesis in Escherichia coli B/r: An analysis of a shift-up. I. Ribosomal RNA chain growth rates. J. Mol. Biol. 75:145-159.

_____and K. Mueller. 1968. Rate of synthesis of messenger ribonucleic acid in Escherichia coli. J. Mol. Biol. 38:329-353.

Buchwald, M., and R. J. Britten. 1953. Incorporation of ribonucleic acid bases in metabolic pool and RNA of E. coli. Biophys. J. 3:155-166.

Cashel, M. 1969. The control of ribonucleic acid synthesis in Escherichia coli. J. Biol. Chem. 244:3133-3141.

Cashel, M. 1973. Personal communication.

_____and J. Gallant. 1968. Control of RNA synthesis in Escherichia coli. I. Amino acid dependence of the synthesis of the substrates of RNA polymerase. J. Mol. Biol. 34:317-330.

_____and _____. 1969. Two compounds implicated in the function of the RC gene in Escherichia coli. Nature 221:838-841.

_____and B. Kalbacher. 1970. The control of ribonucleic acid synthesis in Escherichia coli. J. Biol. Chem. 245:2309-2318.

Coffman, R. L., T. E. Norris, and A. Koch. 1971. Chain elongation rate of messenger and polypeptides in slowly growing Escherichia coli. J. Mol. Biol. 60:1-19.

Edlin, G., and J. Neuhard. 1967. Regulation of nucleoside triphosphate pools in Escherichia coli. J. Mol. Biol. 24:225-230.

_____and G. S. Stent. 1969. Nucleoside triphosphate pools and the regulation of RNA synthesis in E. coli. Proc. Nat. Acad. Sci. 62:475-482.

Ehrenfeld, E. R., and A. L. Koch. 1968. RNA synthesis in penicillin spheroplasts of Escherichia coli. Biochem. Biophys. Acta. 169:44-57.

Franzen, J. S., and S. B. Binkley. 1961. Comparison of the acid-soluble nucleotides in Escherichia coli at different growth rates. J. Biol. Chem. 236:515-519.

Gallant, J., and M. Cashel. 1967. On the mechanism of amino acid control of ribonucleic acid biosynthesis. J. Mol. Biol. 25:545-553.

_____and B. Harada. 1969. The control of ribonucleic acid synthesis in Escherichia coli. J. Biol. Chem. 244:3125-3132.

_____, J. Irr, and M. Cashel. 1971. The mechanism of amino acid control of guanylate and adenylate biosynthesis. J. Biol. Chem. 244:5812-5816.

Goldstein, O. B., B. J. Brown, and A. Goldstein. 1960. Amino acid starvation in an Escherichia coli auxotroph: II. Acid-insoluble purine and pyrimidine derivatives in normal and starved cells. Biochem. Biophys. Acta 43:55-61.

Gros, F., and F. Gros. 1958. The role of amino acids in the synthesis of nucleic acids in Escherichia coli. Exp. Cell. Res. 14:104-131.

Julien, J., R. Rosset, and R. Monier. 1968. The metabolism of ribonucleic acids in Escherichia coli starved for phosphate. Bull. Soc. Chem. Biol. 49:131-145.

Kaempfer, R. O., and B. Magasanik. 1967. Effect of infection with T-even phage on inducible synthesis of beta-galactosidase in Escherichia coli. J. Mol. Biol. 27:453-468.

Kjeldgaard, N. O., O. Maaloe, and M. Schaechter. 1958. Dependency on medium and temperature on cell size and chemical composition during balanced growth of Salmonella typhimurium. J. Gen. Microbiol. 19:592-606.

Koch, A. L. 1965. Kinetic evidence for a nucleic acid which regulates RNA biosynthesis. Nature 205:800-802.

_____ 1968. The evaluation of the rates of biological processes from tracer kinetic data: II. RNA metabolism in growing bacteria. J. Theoret. Biol. 18:105-132.

_____and T. E. Norris. 1973. Personal communication.

Kurland, C. G., and O. Maaloe. 1962. Regulation of ribosomal and transfer RNA synthesis. J. Mol. Biol. 4:193-210.

Morris, D. W., and J. A. DeMoss. 1966. Polysome transitions and the regulation of ribonucleic acid synthesis in Escherichia coli. Proc. Nat. Acad. Sci. 56:262-268.

Neidhardt, F. C. 1963. Properties of bacterial mutant lacking amino acid control of RNA synthesis. Biochem. Biophys. Acta 68:365-379.

_____and L. Eidlic. 1963. Characterization of RNA formed under conditions of relaxed amino acid control in E. coli. Biochem. Biophys. Acta 68:380-388.

_____and O. G. Fraenkel. 1961. Metabolic regulation of RNA synthesis in bacteria. Cold Spring Harbor Symp. Quant. Biol. 26:63-70.

Nierlich, D. P. 1968. Amino acid control over RNA synthesis: A re-evaluation. Proc. Nat. Acad. Sci. 60:1345-1352.

_____and W. Vielmetter. 1968. Kinetic studies on the relationship of ribonucleotide precursor pools and ribonucleic acid synthesis. J. Mol. Biol. 32:135-147.

_____ 1972. The regulation of ribonucleic acid synthesis in growing bacterial cells: I. Control over the total rate of RNA synthesis. J. Mol. Biol. 72:751-764.

Norris, T. E. 1970. Controls affecting RNA synthesis in a chemostat system of Escherichia coli involving different growth rates. Ph.D. Thesis, Indiana University.

_____and A. L. Koch. 1972. Effect of growth rate on the relative rates of synthesis of messenger ribosomal and transfer RNA in Escherichia coli. J. Mol. Biol. 64:633-649.

Novick, A., and L. Szilard. 1950. Description of the chemostat. Exp. Cell. Res. 1:110-118.

Pardee, A., and L. Prestidge. 1956. The dependence of nucleic acid synthesis on the presence of amino acids in Escherichia coli. J. Bact. 71:677-683.

Raina, A., M. Jansen, and S. S. Cohen. 1967. Polyamines and accumulation of ribonucleic acid in some polyauxotrophic strains of Escherichia coli. J. Bact. 94:1684-1696.

Randerath, K., and E. Randerath. 1964. Ion exchange chromatography of nucleotides on poly-(ethyleneimine)-cellulose thin layers. J. Chromatog. 16:111-125.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1957. The studies of biosynthesis in Escherichia coli. 2nd Ed., Carnegie Institute, Washington, D. C.

Ron, E. Z., T. Kohler, and B. D. Davis. 1966. Specific stimulation of RNA synthesis by methionine in several strains of Escherichia coli. J. Mol. Biol. 21:13-27.

Rosset, R., J. Julien, and R. Monier. 1966. Ribonucleic acid composition in bacteria as a function of growth rate. J. Mol. Biol. 18:308-320.

Sands, M. D., and R. B. Roberts. 1952. The effects of tryptophan-histidine deficiency in a mutant of Escherichia coli. J. Bact. 63:505-511.

Shehata, T. E., and A. G. Marr. 1971. Effect of nutrient concentration on the growth of Escherichia coli. J. Bact. 107:210-216.

Smith, R. C., and O. Maaloe. 1964. Growth rate on acid-soluble nucleotide composition of Salmonella typhimurium. Biochem. Biophys. Acta 86:229-234.

Stent, G. A., and S. Brenner. 1961. A genetic locus for the regulation of ribonucleic acid synthesis. Proc. Nat. Acad. Sci. 47:2005-2014.

Taylor, A. L., and C. O. Trotter. 1967. Revised linkage map of Escherichia coli. Bact. Rev. 31:332-353.

Winslow, R. M., and R. A. Lazzarini. 1969. The rate of synthesis and chain elongation of ribonucleic acid in Escherichia coli. J. Biol. Chem. 244:1128-1137.

_____and _____. 1970. The regulation of RNA synthesis during growth rate transitions and amino acid deprivation in E. coli. Cold Spring Harbor Symp. Quant. Biol. 35:383-390.

Wong, J., and R. Nazar. 1972. Nucleotide changes and the regulation or ribonucleic acid accumulation during growth rate shifts in Escherichia coli. J. Biol. Chem. 247:790-797.